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(54) Title: HEPARIN FRAGMENTS AS INHIBITORS OF SMOOTH MUSCLE CELL PROFILERATION

(57) Abstract

A glycosaminoglycoside (GAG) composition predominantly of tetrasaccharide units derived from heparin/heparan sulfate has enhanced activity in preventing the proliferation of smooth muscle cells. This GAG preparation is useful in the treatment of diseases characterized by unwanted smooth muscle cell proliferation and activity.

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HEPARIN FRAGMENTS AS INHIBITORS OF
SMOOTH MUSCLE CELL PROLIFERATION

10

Technical Field

The invention relates to the use of carbohydrate preparations as therapeutic and diagnostic compositions.

15 In particular, the invention relates to a heparin derivative having a molecular weight corresponding to a tetrasaccharide unit to treat diseases and conditions characterized by excessive smooth muscle cell proliferation.

20 Background Art

Proliferation of smooth muscle cells in blood vessel walls occurs in response to vascular injury, and in association with certain disease states (Austin, G.E., et al., J Am Coll Cardiol (1985) 6:369-375). The proliferation of these cells can have negative effects due to the production of excess proteins or other matrix molecules, which, along with the cells themselves, form pathologic lesions of, for example, atherosclerosis, renal hypertension, pulmonary hypertension, vasculitis, and post-surgical vascular retinosis. These results are distinguished from the acute response to trauma characterized by blood clotting.

Glycosaminoglycans (GAG) are alternating copolymers of a hexosamine and an aldouronic acid which are found in sulfated forms and are synthesized as proteo-

35

glycans. They have collectively been called mucopoly-
saccharides. To place the compositions discussed below in
context, it may be noted that heparin and heparan sulfate
are members of the GAG family which are classified by the
5 nature of the hexosamine/aldouronic acid repeating units.
For example, in chondroitin sulfates, the aldouronic acid
is primarily D-glucuronic acid, and the hexosamine is
acetylated 2-amino-2-deoxy-D-galactose (N-acetyl galac-
tosamine, GalNAc). In dermatan sulfate (chondroitin
10 sulfate B) the aldouronic acid is mostly L-iduronic acid
and the hexosamine is GalNAc. In keratin sulfate, the
aldouronic acid is replaced by D-galactose, and the
hexosamine is mostly acetylated 2-amino-2-deoxy-D-glucose
(N-acetyl glucosamine, GlcNAc). In the compositions of
15 interest herein, heparan sulfate and heparin, the hexo-
samine is mostly acetylated and sulfated glucosamine
(GlcNH₂), and the aldouronic acid is mostly L-iduronic in
heparin and mostly D-glucuronic acid in heparan sulfate.
Heparan sulfate is commonly considered to have a higher
20 proportion of glucuronic acid than heparin.

Problems of heterogeneity in preparations of
heparan sulfate or heparin isolated from tissues make
sharp distinctions difficult, since these oligosaccharides
are related by the biosynthesis pathway, as explained
25 below. Conventional heparin (used as an anti-coagulant)
has a molecular weight of 5-25 kd and is extracted as a
mixture of various chain lengths by conventional proced-
ures. These procedures involve autolysis and extraction
of suitable tissues, such as beef or porcine lung, intes-
30 tine, or liver, and removal of nonpolysaccharide compon-
ents.

The molecular weight of the chains in the
extract is significantly lower than the 60-100 kd known to
exist in the polysaccharide chains of the heparin proteo-
35 glycan synthesized in the tissue. The GAG moiety is syn-
thesized bound to a peptide matrix at a serine residue

through a tetrasaccharide linkage region of the sequence Xyl-Gal-Gal-D-GlcA-, which is then elongated at the xylose residue with alternate additions of GlcNAc and D-glucuronic acid. The polysaccharide sidechains are
5 modified by a series of enzymes which sequentially deacetylate the N-acetyl glucosamine and replace the acetyl group with sulfate, epimerize the hydroxyl at C5 of the D-glucuronic acid residue (to convert it to L-iduronic acid and the GAG chain from the heparan type to a heparin
10 type), sulfate the O-2 of the resulting L-iduronic acid and then sulfate the O-6 of the glucosamine residue. Some of the chains are further sulfated at the O-3 of the glucosamine residue, either at the heparan or heparin stage. This further sulfation is associated with the ac-
15 tive site for antithrombin (anticlotting) activity. Other chemically possible sulfation sites are on the O-3 of L-iduronic or D-glucuronic and O-2 of D-glucuronic acid; however, these are seldom found.

Due to their obvious chemical similarity, iso-
20 lated "heparin" may contain considerable amounts of what might otherwise be classified as heparan sulfate.

There is an extensive art concerning depolymerization of heparin/heparan sulfate chains and separation of products by size. Particularly relevant is
25 the report of Guo, Y. et al., Anal Biochem (1988) 168:54-62 which discloses the results of structure determination after the 2,5-anhydromannose at the reducing terminus is reduced to the corresponding 2,5-anhydromannitol.

The following tetrasaccharides were listed
30 specifically by Guo. In these representations, the following abbreviations are used: D-glucuronic acid = GlcA; L-iduronic acid = IdoA; D-glucosamine = GlcNH₂; N-acetyl-D-glucosamine = GlcNAc; D-glucosamine N-sulfate = GlcNS; 2,5-anhydromannose = Man(2,5); 2,5-anhydromannitol
35 = ManH(2,5). The location of the O-linked sulfate residues is indicated by "S" and the number of the

position of sulfation where the SO_3R residue is linked to oxygen. In the designations below, the alpha and beta anomeric linkages are as those conventionally found in heparin and the indicated D or L configurations as set forth above pertains. The locations of the sulfates are shown below the abbreviation for the sugar to which they apply.

10	GlcA - GlcNAc - GlcA - ManH(2,5);
	IdoA - GlcNAc - GlcA - ManH(2,5);
	IdoA - GlcNAc - GlcA - ManH(2,5);
15	2S
	IdoA - GlcNAc - GlcA - ManH(2,5);
	6S
20	GlcA - GlcNAc - GlcA - ManH(2,5);
	6S
	IdoA - GlcNAc - GlcA - ManH(2,5);
	6S 3S
25	IdoA - GlcNAc - GlcA - ManH(2,5);
	6S 6S
	IdoA - RC - IdoA - ManH(2,5);
30	2S 6S 2S
	IdoA - GlcNAc - GlcA - ManH(2,5);
	6S 3S,6S
35	IdoA - RC - IdoA - ManH(2,5)
	6S 2S 6S

(RC represents the ring contracted form analagous to ManH(2,5); it is believed this form is formed when the resulting intermediate hemiacetal is reduced.)

The involvement of heparin or heparan sulfate or
5 degradation products thereof in smooth muscle
proliferation has been recognized for some time. Heparin
and heparan sulfate can slow or arrest the vascular
proliferation associated with injury described hereinabove
(Clowes, A.W., et al., Nature (1977) 265:625-626). The
10 effect of heparan sulfate and heparin on smooth muscle
proliferation is also described by Marcum, J.A., et al. in
Biology of Proteoglycan, Academic Press (1987) pp. 301-
343. The inhibition of vascular smooth muscle cell growth
by heparin was further described by Castellot, J.J. Jr.,
15 et al., J Biol Chem (1982) 257:11256-11260 and the effect
of heparin on vascular smooth muscle cell growth in fetal
tissue was described by Benitz, W.E., et al., J Cell
Physiol (1986) 127:1-7. The effect of heparin as an in-
hibitor of both pericyte and smooth muscle cell prolifera-
20 tion was shown by Orlidge, A., et al., Microvascular
Research (1986) 31:41-53, and these authors further showed
that chondroitin sulfate, and dermatan sulfate do not have
this effect. A review of the effects of heparin and
heparan sulfate on the proliferation of smooth muscle
25 cells is in press by Benitz, W.E. in "The Pulmonary
Circulation: Normal and Abnormal", Fishman, A.P., ed.,
University of Pennsylvania Press (1988).

It is not clear by what mechanism these glyco-
saminoglycans operate, or to what extent they interact
30 with other growth factors such as epithelial and fibro-
blast growth factors. It has been proposed that a 3-O
sulfate on an oligosaccharide of at least 5 sugars is
important in this process (Castellot et al., J Cell Biol
(1986) 102:1979-1984.

35 It has now been found that an enhanced antipro-
liferative activity with respect to smooth muscle cells is

associated with a smaller oligosaccharide portion of the heparin or heparan sulfate GAGs.

Disclosure of the Invention

5 The invention provides a low molecular weight glycosaminoglycan (GAG) composition which has superior specific antiproliferative activity with regard to smooth muscle cells. The existence of this activity in a low molecular weight GAG provides the opportunity for effective pharmaceutical compositions which can be prepared by
10 isolation of the composition from natural sources or, once the precise structure of the GAG is known, synthetically.

 Accordingly, in one aspect, the invention is directed to a process to prepare an GAG subunit of
15 heparin/heparan sulfate having antiproliferative activity. The process comprises separating according to size the components of a mixture formed from the virtually complete depolymerization of heparin/heparan sulfate, and recovering the portion corresponding to a molecular weight
20 characteristic of tetrasaccharides. The invention is also directed to the GAG composition so obtained.

 In other aspects, the invention is directed to antibodies, including monoclonal antibodies, which are immunospecific for the GAG composition of the invention,
25 and to methods to determine the level of active GAG by reaction with these antibodies. In other aspects, the invention is directed to therapeutic compositions of the GAG preparation or of its antibodies which are useful in regulating smooth muscle cell proliferation.

30 In still another aspect the invention relates to a method to separate low molecular weight organic salts from their non-dialyzable inorganic salt contaminants which comprises adsorbing the organic salt onto an ion exchange column to the exclusion of the inorganic salt,
35 and eluting with a dialyzable salt.

Brief Description of the Drawings

Figure 1 shows the steps in the biosynthesis of heparan sulfate and heparin and their relationship to each other.

5 Figure 2 shows representative elution profiles of depolymerized heparin preparations chromatographed on a Biogel P2 gel filtration column.

Figure 3 shows the antiproliferative activity of the compositions of the invention in an in vitro assay.

10

Modes of Carrying Out the Invention

By "heparin/heparan sulfate" is meant a preparation obtained from tissues in a manner conventional for the preparation of heparin as an anticoagulant or otherwise synthesized and corresponding to that obtained from tissue. This preparation may include residues of D-glucuronic acid (GlcA), as characteristic of heparan sulfate as well as iduronic acid (IdoA) as characteristic of heparin. As described in the Background section above, 20 the conversion of D-glucuronic acid to L-iduronic acid is a result of epimerization at the 5 carbon in a heparan-type intermediate. Figure 1 shows the sequence of steps, and reference to Figure 1 will clarify this relationship. To the extent that full conversion has not been made, 25 heparan sulfate characteristics remain in the preparation. Because the precise nature of the polymeric chains in the preparations of heparin is not generally determined, and varies from preparation to preparation, the term "heparin/heparan sulfate" is intended to cover the range of 30 mixtures encountered.

The "heparin/heparan sulfate" preparation can be obtained from a variety of mammalian tissues, including, if desired, human tissue. Generally, porcine or bovine sources are used, and vascular tissue is preferred. A 35 preferred source of heparin/heparan sulfate starting material is porcine intestinal mucosa, and preparations

labeled "heparin" prepared from this tissue source are commercially available. In general, the heparin/heparan sulfate starting material is prepared from the selected tissue source by allowing the tissue to undergo autolysis and extracting the tissue with alkali, followed by coagulation of the protein, and then precipitation of the heparin-protein complex from the supernatant by acidification. The complex is recovered by reprecipitation with a polar nonaqueous solvent, such as ethanol or acetone or their mixtures, and the fats are removed by extraction with an organic solvent such as ethanol and proteins by treatment with a proteolytic enzyme, such as trypsin. Suitable procedures for the preparation of the heparin starting material are found, for example, in Charles, A.F., et al., Biochem J (1936) 30:1927-1933, and modifications of this basic procedure are also known, such as those disclosed by Coyne, E., in Chemistry and Biology of Heparin, Elsevier Publishers, North Holland, New York, Lunblad, R.L., et al., eds. (1981).

Preferably, the heparin/heparan sulfate preparation used as a starting material is first purified by extraction with a solvent in which the heparin is insoluble, such as ethanol or acetone. The purified starting material is then depolymerized.

The depolymerization can use various reagents, such as nitrous acid, heparinase or periodate, preferably nitrous acid. The digestion products of the invention correspond to those obtained when nitrous acid digestion is carried to completion under acid conditions. In typical procedures, the nitrous acid is prepared in situ by solution of sodium nitrite in cold acidic solution at a concentration of 0.01-1.0 M, and the reagent is used to treat the heparin at a concentration of 10-100 mg/ml, at a pH of 1-2, preferably 1.5. The reaction is conducted at room temperature, and when complete, can, if desired, be neutralized by addition of a suitable reagent. The

complete digestion results in a uniquely constituted mixture of components clearly differing from the results of only partial digestion.

Thus, it is clear that the composition of the various sized fractions of a digestion mixture will depend on the nature and degree of digestion. Depending on the digestion method, the resultant type of cleavage at the linkage cleaved may differ, as well as the locations of cleavage as between, for example, linkages involving L-iduronic acid versus glucuronic acid or linkages between sugars of various levels of sulfation. Accordingly, a predominantly tetrasaccharide mixture retrieved will have a different composition when cleavage is by heparinase than when cleavage is by nitrous acid, and will have a different composition as a result of partial depolymerization as opposed to complete depolymerization.

By "complete depolymerization" is meant a degree of depolymerization which is substantially equivalent to the degree of depolymerization which results from the conduct of the depolymerization process as carried out in the procedure described herein in Example 1. Alternate heparin/heparan sulfate sources may be used, and other depolymerization reagents may be used, so long as the depolymerization results in the components obtained when the digestion is conducted using this procedure and as long as the composition having enhanced antiproliferative activity is obtained.

Thus, other depolymerization methods can be used as long as they produce these active components.

The depolymerization results in a mixture of fragments that can then be separated on the basis of size. A variety of size separation techniques is available, including gel permeation, electrophoresis, and thin layer chromatography, density gradient centrifugation; especially preferred is gel filtration chromatography using a Sephadex or polyacrylamide gel system with a fraction-

ation range of about 100-1800 daltons. A particularly preferred gel permeation resin is Biogel P2, and upon separation using this method, fragments containing disaccharides are effectively separated from those containing tetrasaccharides, hexasaccharides, octasaccharides, and polysaccharides of higher molecular weights.

The size separation can be conducted either before or after the reaction mixture is desalted. It has not previously been possible to effect removal of inorganic ions prior to size fractionation because the dialysis membranes previously available failed to retain the low molecular weight disaccharides and tetrasaccharides obtained in the depolymerization reaction. Accordingly, the salt was removed in the size separation procedure itself, e.g., by elution of the adsorbed materials with volatile solvents such as acetic acid.

It has now been found that inorganic ions may be removed by dialysis prior to size separation, or, in the alternative, salt can be used as an elution medium in the size separation. In part, this possibility is due to the recent availability of dialysis membranes which can separate the tetrasaccharides or disaccharides from ions in the size range of chloride ion. These membranes cannot be used directly because the contaminating ions are sulfate ions and the membranes also retain sulfate--i.e., these membranes are not able to separate sulfate ions from tetrasaccharides. Accordingly, if sulfate ion could be removed from the reaction mixture, and replaced by, for example, chloride ion, dialysis can be utilized to remove these smaller inorganic ions.

This is accomplished by first adsorbing the reaction mixture to an anion exchange resin such as DEAE. This is followed by washing and elution with a low molecular weight salt capable of passing through the dialysis membrane, such as sodium chloride. The eluted

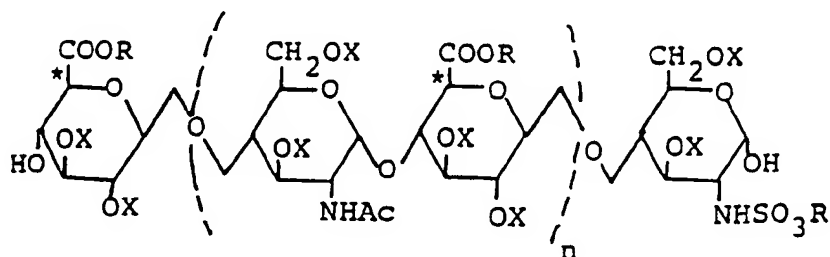
fractions can then be dialyzed with the improved membranes. Accordingly, pharmaceutically acceptable compositions are more readily prepared with an efficient means for salt removal.

5 The fraction containing predominantly tetrasaccharide units shows enhanced activity in inhibiting the proliferation of smooth muscle cells. Verification of this property can be obtained using standard assays, such as those described in Castellot, J.J. Jr., et al., J Cell Biol (1986) 102:1979-1984. Other assay methods, such as those of Benitz, W.E., et al., J Cell Physiol (1986) 127:1-7 (supra) can also be used.

Compounds of the invention which can be thus obtained are of the formula:

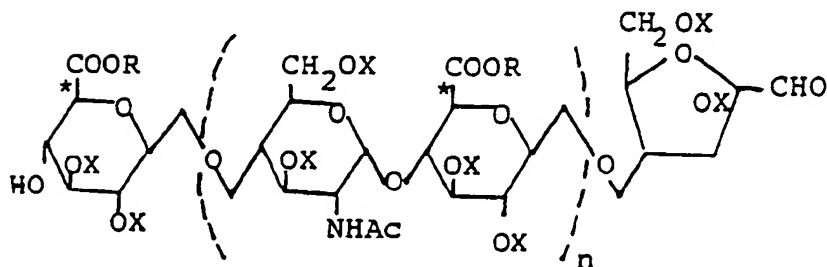
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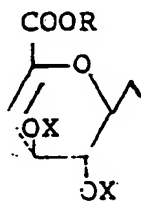
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wherein n is 0 - 2, R is H or a cation, X is H or SO_3R , and Ac is acyl (2-5C), preferably acetyl (Ac^+), and * indicates that the associated C (carbon 5) can be in either the R or S configuration. Saccharide structures

having an odd number of saccharide residues wherein either terminal saccharide is deleted are also included.

In the compounds of formula (2) the sugar at the reducing terminus is deaminated to form the 2,5-anhydromannose shown. When this compound is further reduced, the CHO shown becomes $-\text{CH}_2\text{OH}$; however, this reduction does not occur in the depolymerization reaction per se. Forms wherein the non-reducing terminal sugar is in the 4,5-unsaturated form, i.e.,



may also be included, but do not result from digestion with nitrous acid. Digestion with nitrous acid is specific for cleavage at an N-sulfated glucosamine, therefore complete digestion products of larger chain lengths than disaccharides must contain unsulfated glucosamines.

In particularly preferred embodiments, n is 1. The cations represented by R can either be inorganic cations such as sodium, potassium, calcium, or ammonium ion or can be organic cations such as those obtained from quaternary amines and these salts are formed by simple neutralization.

Representative compounds of the invention, wherein R is as above defined are set forth as follows. In these representations, the following abbreviations are used: D-glucuronic acid = GlcA; L-iduronic acid = IdoA; D-glucosamine = GlcNH₂; N-acetyl-D-glucosamine = GlcNAc; D-glucosamine N-sulfate = GlcNS; 2,5-anhydromannose = Man(2,5); 2,5-anhydromannitol = ManH(2,5). The location of the O-linked sulfate residues is indicated by "S" and the number of the position of sulfation where the SO₃R

residue is linked to oxygen. In the designations below, the alpha and beta anomeric linkages are as those shown in formula 1 above and the indicated D or L configurations as set forth above pertains. The locations of the sulfates are shown below the abbreviation for the sugar to which they apply.

	GlcA	-	GlcNAc	-	GlcA	-	GlcNS;
10	GlcA	-	GlcNAc	-	GlcA	-	GlcNS; 6S
	GlcA	-	GlcNAc	-	GlcA	-	GlcNS; 6S
15	GlcA	-	GlcNAc	-	GlcA	-	GlcNS; 6S
	GlcA	-	GlcNAc	-	GlcA	-	GlcNS; 6S
20	GlcA	-	GlcNAc	-	GlcA	-	GlcNS; 3S,6S
	GlcA	-	GlcNAc	-	IdoA	-	GlcNS; 6S
25	GlcA	-	GlcNAc	-	IdoA	-	GlcNS; 6S
	GlcA	-	GlcNAc	-	IdoA	-	GlcNS; 6S
30	GlcA	-	GlcNAc	-	IdoA	-	GlcNS; 2S
	GlcA	-	GlcNAc	-	IdoA	-	GlcNS; 2S
35	IdoA	-	GlcNAc	-	GlcA	-	GlcNS;

	IdoA	-	GlcNac	-	GlcA	-	GlcNS;
			6S				6S
5	IdoA	-	GlcNac	-	GlcA	-	GlcNS;
	2S		6S				6S
	IdoA	-	GlcNac	-	GlcA	-	GlcNS;
	2S		6S				3S, 6S
10	IdoA	-	GlcNac	-	GlcA	-	GlcNS;
	2S						3S, 6S
	IdoA	-	GlcNac	-	GlcA	-	GlcNS;
	2S		6S				
15	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S		6S		2S		3S, 6S
	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
20	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S						
	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S		6S		2S		6S
25	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S				2S		
	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S				2S		
30	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S		6S		2S		
	IdoA	-	GlcNac	-	IdoA	-	GlcNS;
	2S				2S		6S
35							

	IdoA	-	GlcNAc	-	IdoA	-	GlcNS;
			6S				3S,6S
5	IdoA	-	GlcNAc	-	IdoA	-	GlcNS;
			3S,6S		2S		6S
	IdoA	-	GlcNAc	-	IdoA	-	Man(2,5);
	2S		6S		2S		6S
10	IdoA	-	GlcNAc	-	IdoA	-	Man(2,5);
					2S		6S
	IdoA	-	GlcNAc	-	IdoA	-	Man(2,5);
	2S		6S				
15	IdoA	-	GlcNAc	-	GlcA	-	Man(2,5);
	2S		6S				6S
	IdoA	-	GlcNAc	-	IdoA	-	Man(2,5).
20			3S,6S		2S		6S

Various other levels of sulfation for these representative compounds are also included. In addition, included in the invention, are modified forms of these structures which are disaccharides, trisaccharides, pentasaccharides, and hexasaccharides.

Preparation of Antibodies

The separated composition containing predominantly tetrasaccharide unit fragments can be used to stimulate the production of antibodies which immunoreact with the components of the composition. Standard immunization protocols using this predominantly tetrasaccharide composition in various mammals, such as rabbits, rats, mice, and sheep, result in antisera which are immunoreactive with

the composition components. The composition is advantageously conjugated to a suitable antigenically neutral carrier, such as an appropriate serum albumin or keyhole limpet hemocyanin, in order to enhance immunogenicity.

- 5 Furthermore, the antibody-secreting cells of the immunized mammal can be immortalized to generate monoclonal antibody panels which can then be screened for reactivity with the composition. Techniques for preparation of monoclonal antibodies to heparin oligosaccharides are described by
10 Pejler, G., et al., J Biol Chem (1988) 263:5197-5201, incorporated herein by reference.

The resulting polyclonal or monoclonal antibody preparations are useful in assays for levels of active antiproliferative component in biological samples, as
15 described below, as well as in passive therapy to prevent excessive repression of the proliferation of smooth muscle cells.

Statement of Utility

- 20 The oligosaccharide composition of the invention, containing predominantly tetrasaccharide fragments, is useful in therapeutic applications for treatment of conditions or diseases which are characterized by excessive and destructive smooth muscle cell proliferation.
25 These conditions frequently occur where the subject has been exposed to trauma, such as in the case of surgical patients. The trauma caused by wounds or surgery results in vascular damage and secondary smooth muscle cell proliferation, which secondary proliferation results in
30 vascular retinosis. This undesirable result can occur after vascular graft surgery, heart transplantation, balloon or laser angioplasty, arterial traumatic injury, postsurgical repair of muscular arteries, long-term indwelling of arterial catheters, invasive arterial diagnostic procedures, kidney, lung or liver transplants,
35 coronary artery bypass surgery, carotid artery bypass

surgery, femoral popliteal bypass surgery, and intracranial arterial bypass surgery.

In addition to secondary smooth muscle cell proliferation events occurring as a result of trauma, certain diseases are associated with unwanted vascular proliferation, although in these cases, too, it is assumed that some internal unknown injury has caused the secondary result. These disease states include Goodpasture syndrome, acute glomerulonephritis, neonatal pulmonary hypertension, asthma, congestive heart failure, adult pulmonary hypertension, and renal vascular hypertension.

For all these diseases and conditions, administration of suitable amounts of the composition of the invention is useful in treatment. Administration is by typical routes appropriate for polysaccharide compositions, and generally includes systemic administration, such as by injection. Particularly preferred is intravenous injection, as continuous injection over long time periods can be easily continued. Typical dosage ranges are in the range of 0.1-10 mg/kg/hr on a constant basis over a period of 5-15, preferably 7-10, days. Particularly preferred dosage is about 0.5 mg/kg/hr, or, for a 70 kg adult, 35 mg/hr or 840 mg/day.

Other modes of administration are less preferred but may be more convenient. Injection subcutaneously at a lower dose or orally at a slightly higher dose than intravenous injection, or by transmembrane or transdermal or other topical administration for localized injury may also be effective. Localized administration through a continuous release device, such as a supporting matrix, perhaps included in a vascular graft material, is particularly useful where the location of the trauma is accessible.

Formulations suitable for the foregoing modes of administration are known in the art, and a suitable composition formulation is found in Remington's Pharmaceuti-

cal Sciences, Mack Publishing Company, Easton, PA, latest edition.

The composition of the invention may also be labeled using typical methods such as radiolabeling, fluorescent labeling, chromophores or enzymes, and used in a competitive assay for the amount of antiproliferative component in a biological sample. Suitable protocols for competitive assays of analytes in biological samples are well known in the art, and generally involve treatment of the sample, in admixture with the labeled competitor, with a specific binding partner which is reactive with analyte such as, typically, an immunoglobulin or fragment thereof. The antibodies prepared according to the invention are useful for this purpose. The binding of analyte and competitor to the antibody can be measured by removing the bound complex and assaying either the complex or the supernatant for label. The separation can be made more facile by preliminary conjugation of the specific binding partner to a solid support. Such techniques are well known in the art, and the protocols available for such competitive assays are too numerous and too well known to be set forth in detail here.

The antibodies of the invention are useful in immunoassays, not only of the type described above involving competition between labeled composition and the analyte antiproliferation factor in the sample, but also for direct immunoassay for the factor. Alternate protocols involving direct assays are also of wide variety and well known. Typically, the analyte bound to antibody is detected by means of an additional reactive partner which bears a label or other means of detection. Thus, in typical sandwich assays, for example, the binding of the antibodies of the invention to analyte can be detected by further reaction with a labeled preparation of these same antibodies or by labeled antibody immunoreactive with this preparation by virtue of species differences.

The antibodies of the invention can also be formulated into pharmaceutical compositions and used to stimulate the growth of smooth muscle cells in subjects for which this result is desirable.

5 The following example is intended to illustrate but not to limit the invention.

Example 1

Preparation of the

10 Predominantly Tetrasaccharide Composition

Commercial porcine intestinal mucosa heparin was dissolved in 1 M NaCl to approximately 270 mg/ml, and then precipitated by addition of 3 volumes of 95% ethanol. The precipitated heparin was centrifuged at 3,000 x g and the
15 pellet recovered and redissolved and reprecipitated as described for an additional 2 precipitations. The final heparin pellet was then lyophilized.

To prepare the nitrous acid reagent, sodium nitrite was dissolved in ice cold 0.24 M citric acid to
20 obtain a 0.06 M solution of NO_2^- . 5 parts of the nitrous acid reagent was then added to 1 part of a 300 mg/ml heparin solution. The preparation was allowed to come to room temperature, titrated to pH 1.5 with 1 M H_2SO_4 and allowed to react for 1 hr at 30°C. The reaction was
25 quenched by addition of ammonium sulfamate to 0.5 M.

The resulting depolymerized heparin was then applied to a 2.5 x 97 cm Biogel P2 gel filtration column packed in 15% v/v acetic acid. Elution with 15% v/v acetic acid was continued, and fractions were collected.
30 The concentration of uronic acids in each fraction was determined using the carbazole assay of Bitter, T., et al., Anal Biochem (1962) 4:330.

Typical elution profiles are shown in Figure 2. The peak elution at approximately 320 ml ($V_e/V_o = 0.67$)
35 contains mostly tetrasaccharides.

The fraction containing the tetrasaccharide-predominating mixture is then lyophilized for storage.

Example 2

5 Effect on Smooth Muscle Proliferation

Solutions to be tested were made up in "complete medium", which is DMEM medium containing 10% fetal calf serum and penicillin/streptomycin.

Bovine smooth muscle cells (SMC) were isolated
10 from bovine pulmonary artery by the method of Benitz, W.E., et al., J Cell Physiol (1986) 127:1-7. SMC from passage 3-10 were plated at 350-700 cells per well in 96-well microtiter plates in the medium above and allowed to attach for 2-4 hr. The complete medium was replaced
15 with DMEM supplemented with 0.1% fetal calf serum, and the cells were incubated for an additional 72 hr to arrest cell growth. The low-serum medium was then replaced with complete medium containing the test samples.

The cells were allowed to grow for up to 7 days
20 with replicate plates sampled at regular intervals. Cell number was determined by removing the medium and washing the cells with phosphate-buffered saline, adding 75-150 ul lysis buffer, and assaying for lactate dehydrogenase (LDH) activity, as described by Brandley, B., et al., J Biol
25 Chem (1987) 262:6431. The activity of LDH is proportional to cell number.

The results of one such assay are shown in Figure 3. Bars #1 and #5 are controls. Bar #1 contains no GAG. Bar #5 represents a test solution of 150 ug/ml
30 chondroitin sulfate. Bar #4 contains 150 ug/ml of commercial heparin. Bars #2 and #3 contain the composition of the invention at 50 ug/ml. The ability of the invention composition to inhibit the proliferation of growth exceeds that of commercial heparin.

35 At 60 ug/ml, the composition of the invention as prepared in Example 1 inhibits growth of SMC 90%, as com-

pared to controls; at 6.6 ug/ml, it inhibits growth of SMC to a level equal to that obtained with 150 ug/ml heparin, or by 50%.

A second preparation of the invention composition inhibited SMC growth 50% at concentrations of 10-100 ug/ml.

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Claims

1. A method to prepare a glycosaminoglycoside (GAG) composition useful in preventing or inhibiting the proliferation of smooth muscle cells which method comprises:

separating a mixture obtainable by complete nitrous acid depolymerization of mammalian heparin/heparan sulfate into fragments according to molecular size, and recovering the size fraction of the mixture comprised predominantly of fragments which are tetra-saccharide units.

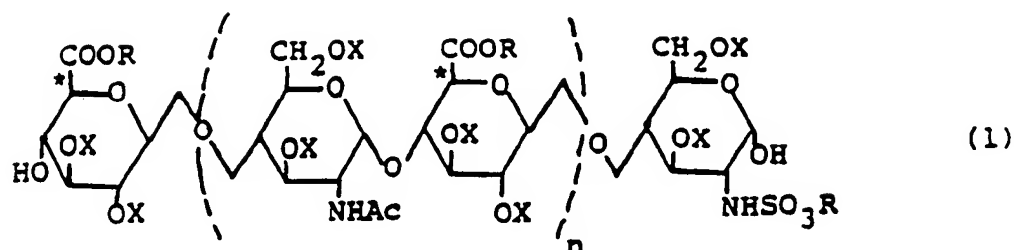
2. The process of claim 1 wherein the fragments are separated by gel filtration conducted using a column containing Biogel P2, and eluting with 15% V/V acetic acid.

3. A GAG composition prepared by the process of claim 1.

4. Antibodies specifically immunoreactive with the oligosaccharide composition of claim 3 and prepared by a process which comprises immunization of a mammal with the said composition.

5. A compound obtainable by isolation from heparin completely depolymerized by nitrous acid digestion which is a hexasaccharide, pentasaccharide, tetrasaccharide, trisaccharide or disaccharide or salt thereof, capable of preventing or inhibiting the proliferation of smooth muscle cells.

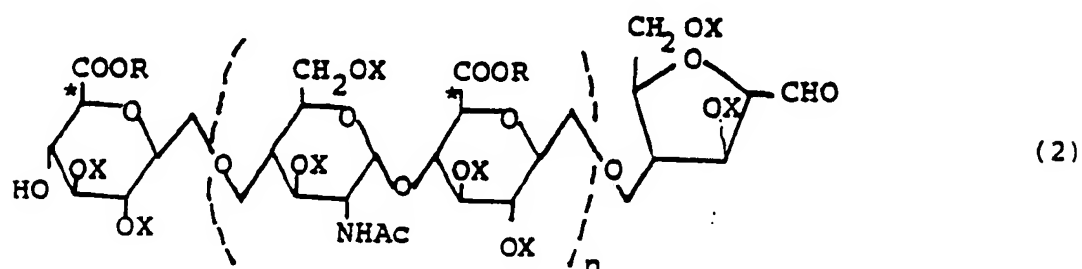
6. The compound of claim 5 which has the formula:



wherein n is 0-2, R is H or a cation, X is H or SO_3R and Ac is acyl (2-5C), and * indicates that the associated C (carbon 5) can be in either the R or S configuration, or the compound of formula (1) wherein the reducing or non-reducing terminal sugar is deleted.

7. The compound of claim 6 wherein $n=1$.

8. The compound of claim 5 which has the formula



wherein n is 0 - 2, R is H or a cation, X is H or SO_3R , and Ac is acyl (2-5C) and * indicates that the associated C (carbon 5) can be in either the R or S configuration of the compound of formula (2) wherein the reducing or non-reducing terminal sugar is deleted.

9. The compound of claim 8 wherein $n=1$.

10. A pharmaceutical composition useful in the treatment of conditions characterized by unwanted smooth muscle cell proliferation, which composition comprises an effective amount of the compound of claims 3 or 5-9 in admixture with at least one pharmaceutically acceptable excipient.

11. A method to diagnose a deficiency or excess in antiproliferative GAG in a mammalian subject which method comprises assessing in a biological sample taken from the subject the level of GAG competitive in an immunoassay with the compound of claim 3 or 5-9 in labeled form.

12. Antibodies specifically immunoreactive with the compound of claim 3 or 5-9 and prepared by a process which comprises immunization of a mammal with said compound.

13. A method to assay the level of antiproliferative factor in a biological sample, which method comprises treating said sample with the antibodies of claim 4 or 12, and detecting the amount of antibodies bound by the sample.

14. A method to separate organic ions from inorganic ions which inorganic ions are retained by a dialysis membrane, which process comprises:

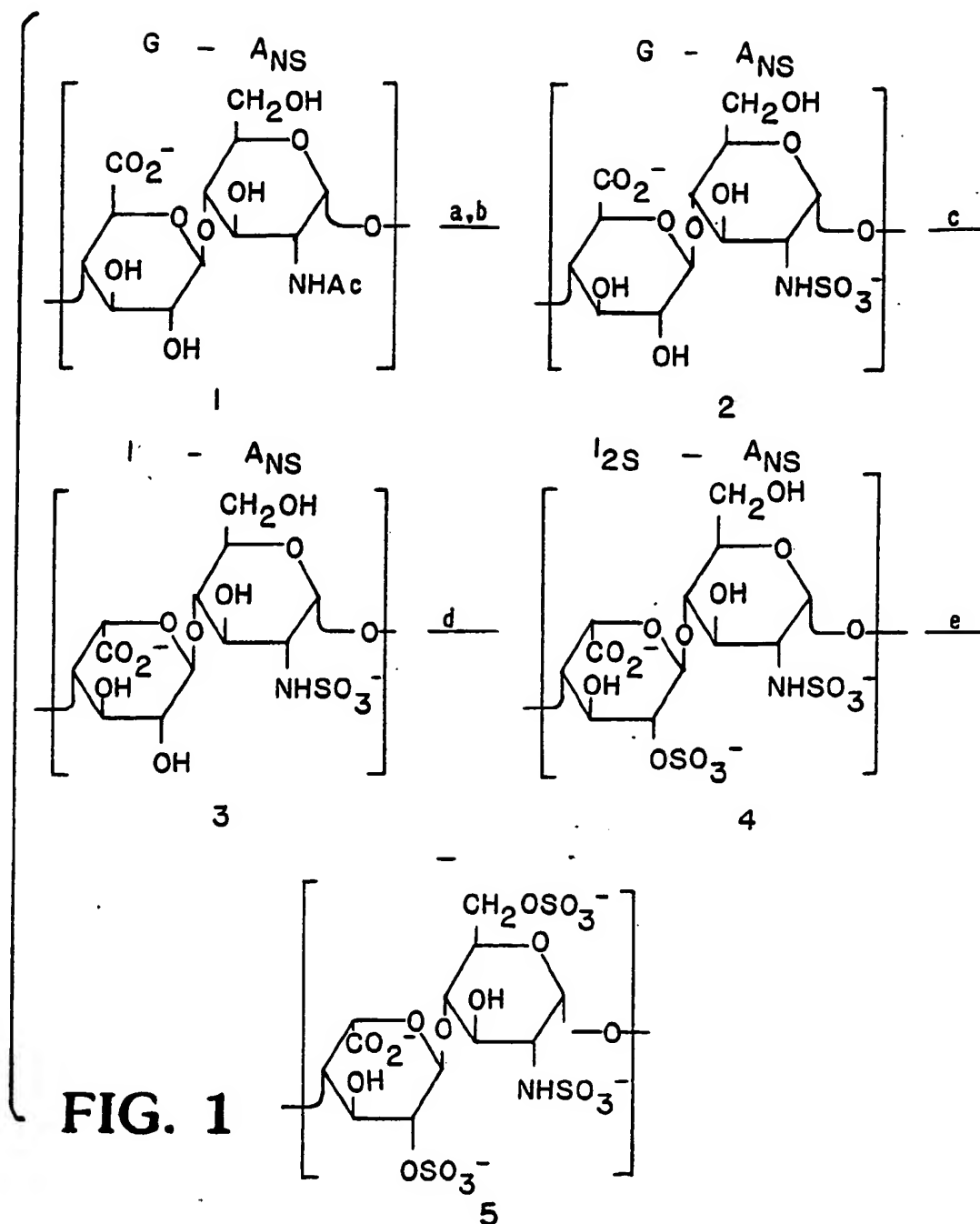
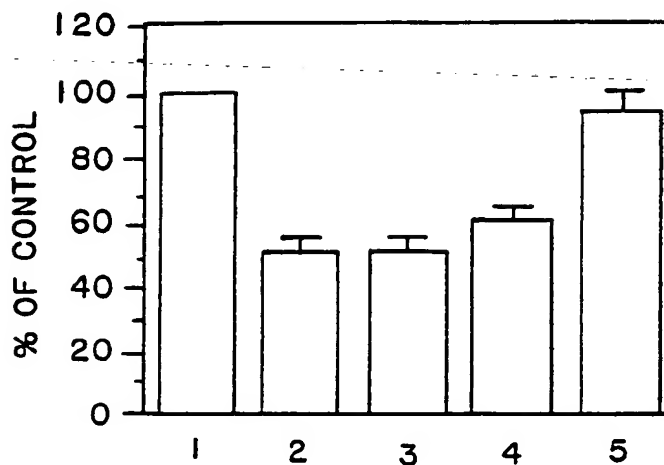
adsorbing the organic ion to an ion exchange resin under conditions wherein the inorganic ion is not adsorbed;

eluting the organic ion from the resin using an organic solvent or a salt comprising an ion which is not retained by a dialysis membrane to obtain eluted fractions; and

subjecting the eluted fractions containing the organic ion to dialysis.

15. The method of claim 14 wherein the inorganic ion is sulfate and the organic ion is a sulfated sugar.

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**FIG. 1****FIG. 3**

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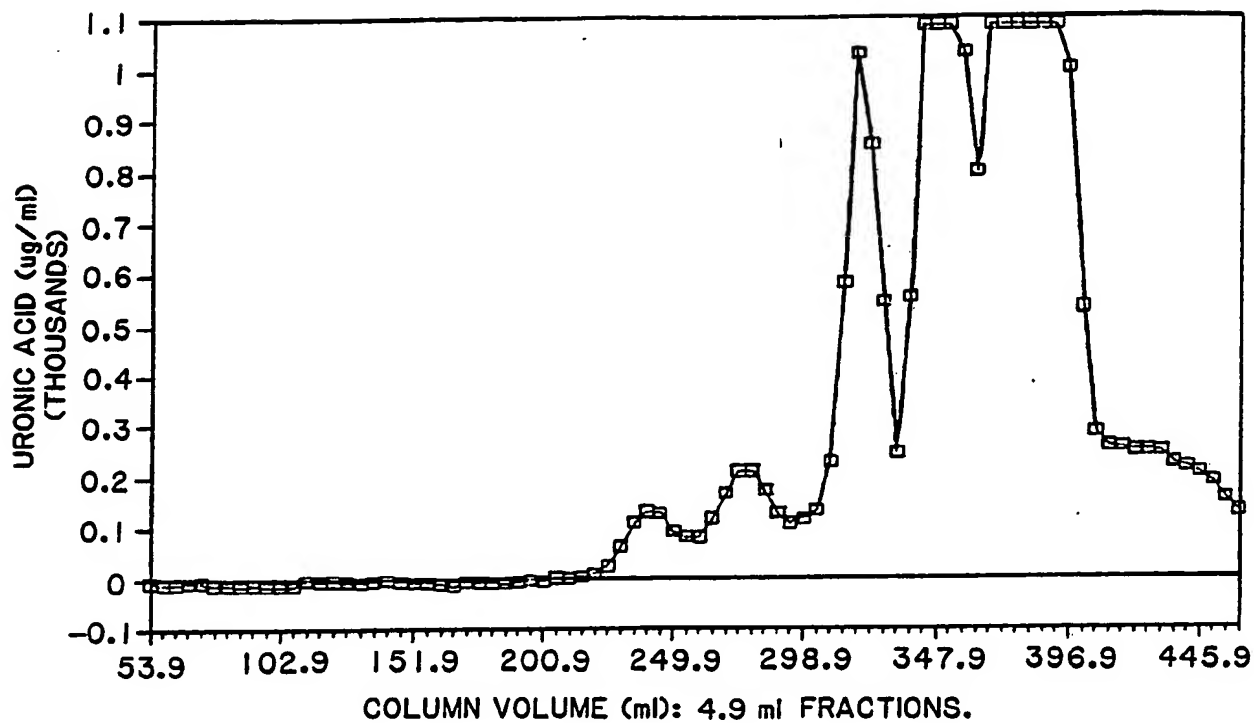
HONO $\text{Ba}(\text{NO}_2)_2$ HEPARIN DIGEST

FIG.2-1

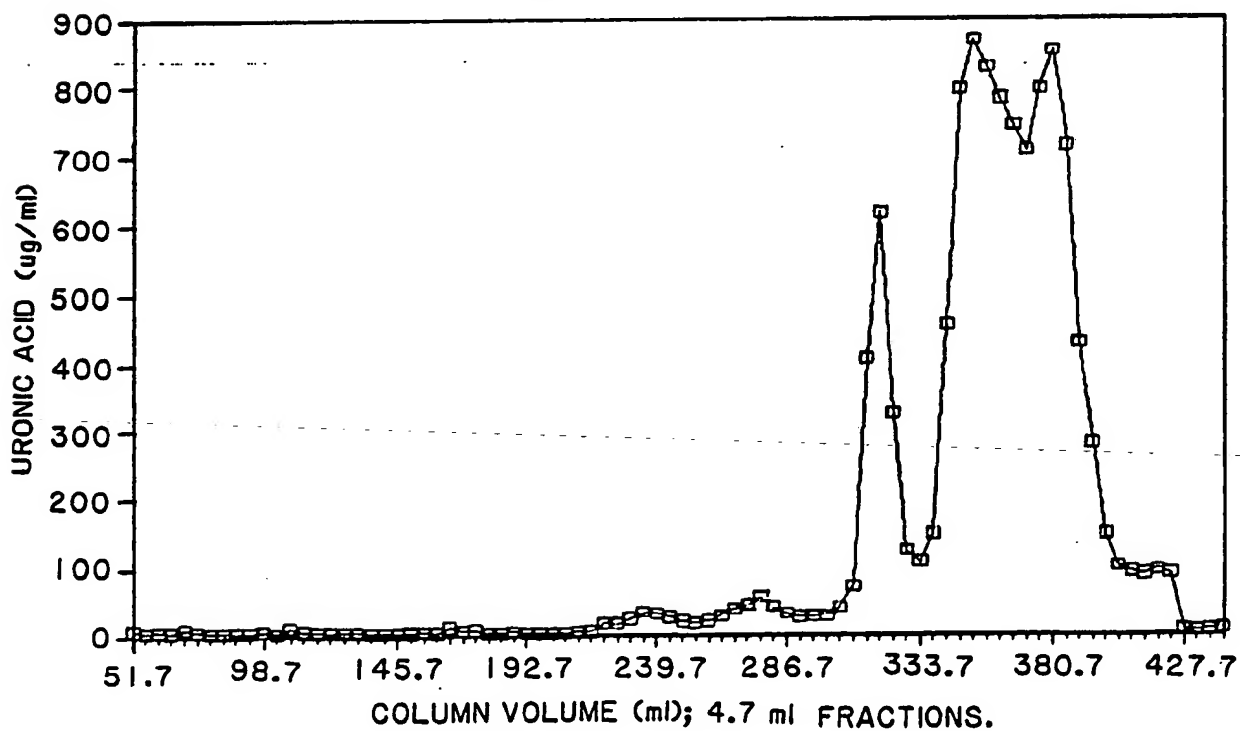
HONO NaNO_2 HEPARIN DIGEST

FIG. 2-2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/05559

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC (5): A61K 31/70, . 31/715 COBB 37/10 U.S. CL. 536/21,124; 436/578, 536
 COIN 33/00; BOLD 15/00 514/56; 530/387; 210/660

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System

Classification Symbols

U.S. CL.

536/21,124; 514/56; 530/387; 210/660; 436/518,536

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
<u>Y</u> <u>A</u>	US, A, 4,401,662, LORMEAU ET AL. 30 AUGUST 1983	1-3, 5-7, 10 8,9
<u>Y</u> <u>A</u>	Casu, (1985) "Structure and Biological Activity of Heparin", Advances in Carbohydrate Chemistry and Biochemistry <u>43</u> : 51-134 (See entire document)	1-3 5-11
<u>Y</u> <u>A</u>	Castellot et al., (1986) Journal of Cell Biology, <u>102</u> : 1979-1984 (See entire document)	11 1-3, 5-10
A	Benitz et al., (1986) Journal of Cellular Physiology 127: 1-7 (See entire document)	1-3, 5-11
<u>Y</u> <u>A</u>	Scott-Burden, et al., TIPS (MARCH 1988) <u>9</u> : 94-98 (See entire document)	1-3

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

13 FEBRUARY 1990

02 APR 1990

International Searching Authority

ISA/US

Signature of Authorized Officer

Everett White
EVERETT WHITE

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

<u>Y</u> A	Marcum, et al., Biology of Proteoglycan, Academic Press, 1987, p. 301-343 (see pages 310-313)	5 1-3, 6-11
A	Castellot, et al. (1982), Journal of Biological Chemistry, 257 (19): 11256-11260 (See entire document)	1-3, 5-11
A	Orlidge, et al. (1986), Microvascular Research, 31: 41-53 (See entire document)	1-3, 5-11

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Pejler, et al. (1988), <u>Journal of Biological Chemistry</u> , <u>263</u> (11): 5197-5201. (See entire document)	1-5, 12, 13
Y	Guo, et al. (1988), <u>Analytical Biochemistry</u> , <u>168</u> : 54-62 (See entire document)	1,2,5,14,15
A	Austin, et al. (1985), <u>J.Amer.Coll. Cardiol.</u> , <u>6</u> : 369-375 (See entire document)	1
A	Clowes, et al. (1977), <u>Nature</u> , <u>265</u> : 625-626 (See entire document)	1,2
A	Benitz, "The Pulmonary Circulation: Normal and Abnormal," Fishman, A.P., ed. University of Pennsylvania Press (1988) (See entire document)	1-3, 5-11

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